1 ***Aspergillus fumigatus* mycovirus causes mild hypervirulent effect on pathogenicity when tested on**

2 ***Galleria mellonella***

3 Selin Özkan* and Robert H. A. Coutts

4 *Department of Life Sciences, Faculty of Natural Sciences, Imperial College London, Sir Alexander Fleming*

5 *Building, Imperial College Road, London, SW7 2AZ, UK*

6

7 *Corresponding author. Fax (+) 44 2075842056

8 E-mail address: s.ozkan10@imperial.ac.uk (S. Özkan)

9

10 **ABSTRACT**

11 Mycoviruses are a specific group of viruses that naturally infect and replicate in fungi. The importance of
12 mycoviruses was revealed after their effects were identified not only in economically important fungi but
13 also in the human pathogenic fungus *Aspergillus fumigatus*. The latter was shown recently to harbor at least
14 three different types of mycoviruses including a chrysovirus, a partitivirus and as yet uncharacterised virus.
15 Assessment of virulence in the presence and absence of mycoviruses in *A. fumigatus* is pivotal to
16 understanding its pathogenicity. Here, we have investigated, for the first time, the effects of mycoviruses
17 on the pathogenicity of *A. fumigatus* as assessed using larvae of the greater wax moth *Galleria mellonella*.
18 In order to observe the effects of mycoviruses on pathogenicity, *G. mellonella* were injected with virus-free
19 and virus-infected isolates of *A. fumigatus* and post-infection survival times were analyzed along with the
20 fungal burden. Neither chrysovirus nor partitivirus infection affected fungal pathogenicity when survival
21 rates were assessed which, for the chrysovirus, agreed with a previous study on murine pathogenicity.
22 However statistically significant differences were observed in survival rates and fungal burden in the
23 presence of the uncharacterized A78 virus. Here we show, for the first time, the effects of a partitivirus and
24 an uncharacterized A78 virus on the pathogenicity of *A. fumigatus*.

25 Keywords: *Aspergillus fumigatus*; dsRNAs; mycoviruses; *Galleria mellonella*; virulence; pathogenicity

26 **1. Introduction**

27 Mycoviruses are a specific group of viruses which naturally infect and replicate in fungi. They are
28 widespread in all major fungal groups, most being latent or cryptic and asymptomatic in their hosts
29 (Ghabrial and Suzuki, 2009). However, there are numerous examples of mycoviruses that alter host
30 phenotype and growth rate and act as hypovirulence factors (Nuss, 2005). Hypovirulence, attenuated
31 pathogenicity in fungi, is characterized by a reduction in conidiation, pigmentation and growth rate (Dawe
32 and Nuss, 2001). Paradoxically, mycoviruses can also confer hypervirulence which is characterized by
33 enhanced sporulation, aggressiveness and growth (Ahn and Lee, 2001). However, the possible role of
34 mycoviruses in regulating the ecology of their host fungi and associated hosts is largely unknown (Hyder et
35 al., 2013).

36 *Aspergillus fumigatus* is an opportunistic fungal pathogen that causes lung disease in humans and
37 animals. While it is responsible for severe invasive aspergillosis in immunocompromised patients, it can lead
38 to allergic reactions and chronic lung disease in immunocompetent individuals (Latge, 1999). Previous
39 research on *A. fumigatus* suggested that the fungus was devoid of double-stranded (ds) RNA elements
40 which comprise the majority of mycoviruses (Varga et al., 1998). However, after screening more than 360
41 *A. fumigatus* isolates from UK environmental and clinical sources for the presence of dsRNA elements, at
42 least three different dsRNA profiles were observed (Bhatti et al., 2012). A similar incidence of dsRNA
43 elements has recently been reported in 18.6% of *A. fumigatus* isolates in Holland (Refos et al., 2013). From
44 the UK cohort, two mycoviruses, nominated *Aspergillus fumigatus* chrysovirus (AfuCV) and *Aspergillus*
45 *fumigatus* partitivirus (AfuPV-1), have been sequenced completely (Jamal et al., 2010; Bhatti et al., 2011a
46 respectively); a novel virus remains partially characterized (Fig. 1).

47 The influence of mycoviruses on the fitness and pathogenicity of *A. fumigatus* was investigated by
48 generating virus-infected and virus-free isogenic lines from isolates originally infected with AfuCV and
49 AfuPV-1 (Bhatti et al, 2011b). Infection of *A. fumigatus* with either the chrysovirus (CV) or the partitivirus
50 (PV) resulted in significant aberrant phenotypic alterations *in vitro* and attenuation of fungal growth but
51 had no effects on susceptibility to common antifungals (Bhatti et al, 2011b). Furthermore, CV infection of

52 *A. fumigatus* caused no effect on pulmonary fungal burden as estimated by quantitative PCR or any
53 significant alterations to murine pathogenicity (Bhatti et al, 2011b).

54 We sought to use a reliable and low-cost model organism to assess *A. fumigatus* pathogenicity. Larvae
55 of the greater wax moth *Galleria mellonella* have been used as a model organism to evaluate the virulence
56 of fungal pathogens and the effects of antimicrobial drugs (Mowlds and Kavangh, 2008). Indeed, several
57 studies have employed *G. mellonella* to assess the pathogenicity of bacteria such as *Pseudomonas*
58 *aeruginosa* (Andrejko et al., 2009) and *Legionella pneumophila* (Harding et al., 2012), yeasts such as
59 *Cryptococcus neoformans* (Mylonakis et al., 2005) and *Candida albicans* (Brennan et al., 2002) and fungi
60 such as *A. fumigatus* (Reeves et al., 2004; Fallon et al., 2011). *G. mellonella* larvae offer a variety of
61 advantages such as temperature range in which they can survive, common characteristics with the
62 mammalian immune system, simplicity of handling, easiness of injection due to size and shortness of the
63 monitoring time of the infections (Fuchs et al., 2010).

64 In this investigation, we aimed to assess the effect of mycoviruses on the pathogenicity of *A. fumigatus*
65 using the *G. mellonella* infection model. In particular, we have studied in detail all the known mycovirus -
66 *A. fumigatus* combinations in a comparative approach with virus-free isogenic lines and with particular
67 reference to the partitivirus and the as yet uncharacterized virus.

68 **2. Materials and methods**

69 *2.1. Aspergillus fumigatus strains and growth conditions*

70 All *A. fumigatus* strains were naturally occurring wild types or were generated following mycovirus
71 transfection (virus-infected) or mycovirus elimination (virus-free). The isolates were confirmed as being
72 *bona fide A. fumigatus* strains following generation of sequence specific ITS amplicons using ITS specific
73 oligonucleotide primers ITS-1 and ITS-4 (Fig. S1) and also DNA sequencing (data not shown). Three different
74 virus-infected and virus-free host combinations were used along with the control, non-virulent *Δpaba*
75 strain, as listed in Table 1. Elimination of virus-infection was achieved using cycloheximide as described
76 previously (Bhatti et al., 2011b). All strains were kept as glycerol stocks at -20 °C. They were inoculated on

77 plates containing Aspergillus complete medium (ACM; Pontecorvo et al., 1953) with 1% agar and incubated
78 at 37 °C. Following 5 days incubation, spores were harvested using Dulbecco's PBS buffer (Invitrogen,
79 Carlsbad, CA) containing 0.05% Tween 80 (Sigma, St. Louis, MO). Spore concentrations were determined
80 using a haemocytometer and spore suspensions were stored at 4 °C for a maximum period of 12 h prior to
81 injection.

82 *2.2. G. mellonella larvae*

83 Final-instar larvae of the greater wax moth (*Galleria mellonella*) were obtained from Livefood UK Ltd.
84 (Somerset, England) and stored prior to use in wood shavings in the dark to prevent pupation. Grey marked
85 larvae were excluded and only cream-colored larvae were used within 1 week after delivery in order to
86 observe pigmentation changes clearly (Fuchs et al., 2010). Ten larvae weighing approximately 0.2 g each
87 were chosen at random and all experiments were duplicated and repeated on three independent occasions.

88 *2.3. Injection of G. mellonella larvae*

89 *G. mellonella* larvae were injected with 10 µl of fungal spore suspension through the last left proleg into
90 the hemocoel using a Hamilton syringe and a 22s gauge needle (Hamilton, USA; Fallon et al., 2012). Ten
91 larvae were employed for each experiment as described in Section 2.2. Control experiments comprised: (i)
92 untouched larvae (UTC), (ii) pierced larvae (PC) (iii) PBS-injected larvae (PBS). The Δ*paba A. fumigatus* strain
93 was used as a non-virulent control.

94 *2.4. Survival assay*

95 In initial experiments, larvae were injected with serially diluted spore suspensions ranging in
96 concentration from $1x10^4$ to $1x10^7$ spores in order to determine the optimal spore concentration to facilitate
97 clear observations of the pathogenicity of virus-free and virus-infected isolates. For survival rate assays,
98 $1x10^5$ spores were inoculated as described in Section 2.3 and infected larvae were incubated in Petri dishes
99 in the dark at 37 °C for 5 days. Their times of death were recorded daily together with notes on any
100 melanization and lack of motility. Survival curves were plotted and their statistical significance were

101 determined by Kaplan-Meier analysis using the GraphPad Prism 6.0 program and *P* values were estimated
102 using Log rank and Wilcoxon tests. *P* values less than 0.05 were accepted as statistically significant.
103
104 *2.5. Radial growth assay of A-78 infected Aspergillus fumigatus isolate*

105 Equal numbers of spores (n=10^3) of isogenic lines of *A. fumigatus*, isolates A78-infected and A78-free
106 were centrally inoculated onto Aspergillus minimal medium (AMM; Barratt et al.,1965) and ACM agar in 90
107 mm Petri plates, incubated at 37 °C and the fungal phenotype examined 5 days after inoculation. The colony
108 diameters of the two isolates were measured every 24 h over the 5 day incubation period in three replicate
109 plates. The means and standard errors of the colony diameters measured were plotted to determine any
110 differences between the isolates. All experiments were performed in triplicate.
111
112 *2.6. Fungal burden assay using quantitative PCR*

113 For the fungal burden assay, moth larvae were treated as for the survival assay. Following 6h and 48 h
114 at 37 °C incubation, three randomly selected larvae for each time point were ground using a mortar and
115 pestle in liquid N_2 and homogenized in 5 ml PBS buffer. In order to assess the fungal burden quantitatively,
116 quantitative PCR (qPCR) was performed on genomic DNA extracted from 200 µl of larval homogenate using
117 the DNeasy Blood and Tissue Kit according to the manufacturer's protocol (Qiagen, Valencia, CA). Fungal
118 burden was determined using the primers βT-F (5'-AATTGGTGCCGCTTTCTGG-3') and βT-R (5'-
119 AGTTGTCGGGACGGAATAG-3') to amplify the *A. fumigatus* beta-tubulin gene (Balajee et al., 2005). The
120 primers Actin-F (5'-ATCCTCACCCTGAAGTACCC-3') and Actin-R (5'-CCACACGCAGCTCATTGTA-3') were used
121 to amplify the *G. mellonella* actin gene (Altincicek and Vilcinskas, 2006). Quantitative PCR assay was
122 performed using SYBR green I (Invitrogen, Carlsbad, CA) as a fluorescent dye and monitored using an ABI
123 Prism 7700 machine (Perkin-Elmer Applied Biosystems, Waltham, MA). Three biological replicates for each
124 isolate were carried out along with a PBS-injected control and *Δpaba*-injected control in MicroAmp 96-well

125 plates (Applied Biosystems, Grand Island, NY). Differences between the values of threshold cycle (Ct) were
126 analyzed using unpaired t-tests (GraphPad Prism version 6.04).

127 **3. Results**

128 *3.1. Determination of the optimal spore concentration of A. fumigatus for pathogenicity testing in G.*
129 *mellonella*

130 All six isolates under investigation exhibited different phenotypes including pigmentation and sectoring
131 when grown on solid ACM (Fig.2). Two contrasting isolates of *A. fumigatus* were selected to determine the
132 appropriate inoculum concentration for the investigation. These were the fastest growing A78-infected
133 isolate and the slowest growing PV-free isolate (Figs. 3A and 3B, respectively). An infectious dose of $1x10^7$
134 spores/larva resulted in 100% mortality within 24-48 h while $1x10^4$ spores/larva was not infectious (Figs. 3A
135 and 3B). A concentration of $1x10^5$ spores/larva was chosen as optimal since its intermediate pathogenicity
136 level facilitated determining differences in virulence between the different isolates. In these experiments,
137 which were duplicated, all control larvae survived until the end of the observation period (120 h).

138 *3.2. Survival rates of G. mellonella larvae following infection with A. fumigatus*

139 All isolates used in this study were highly virulent as compared to the non-virulent *Δpaba* isolate
140 following injection of larvae with $1x10^5$ spores/larva (P<0.0001). Melanization, which is a strong indicator
141 of fungal growth and an active immune response, was observed in all infected larvae within 18 h post-
142 injection except those injected with *Δpaba*. There were no significant differences between the survival rates
143 of CV-free and CV-infected *A. fumigatus* isogenic lines (P=0.87; Fig. 4A). Also, there were no significant
144 differences in the survival rates of *A. fumigatus* PV-free and PV-infected injected moth larvae (P=0.15; Fig.
145 4B).

146 However, there were statically significant difference in the survival rate of the A78-free and A78-infected
147 *A. fumigatus* isogenic lines (P<0.01; Fig. 4C). This suggested that virus-infected A78 *A. fumigatus* isolate was
148 more virulent than the corresponding virus-free isogenic line in *G. mellonella*.

149 *3.3. Radial growth of Aspergillus fumigatus isolate A78*

150 Compared to the A78-free cultures, there were marked alterations in the phenotype associated with
151 virus-infection in the *A. fumigatus* A78 isolate, including deeper pigmentation and a uniform, sectored
152 growth (Fig. 2). The radial growth assays showed that the A78-infected *A. fumigatus* isolate grew faster than
153 the A78-free isolate. This trend was more noticeable and maintained on AMM and occurred at an earlier
154 time (Fig. 5A). In contrast, on ACM, the growth difference was noticeable only on day 4 (Fig. 5B). Thus, the
155 radial growth assays demonstrated that the virus infection caused increased growth on the medium which
156 is similar to that observed in the *G. mellonella* survival assay (Fig. 4C).

157 *3.4. Fungal burden*

158 Quantitative PCR was performed using the expression of the *A. fumigatus* β-tubulin gene as an indication
159 of fungal growth and burden in moth larvae inoculated with all isolates (Table 1). The actin gene that was
160 used as an internal control, remained at the same level in all of the qPCR experiments and, as anticipated
161 for a negative control, there was no β-tubulin gene expression at both time points in all PBS injected larvae
162 (Fig. 6). In *Δpaba* injected larvae, higher Ct values meaning less expression were detected when compared
163 to the virus-free and virus-infected isolates. The expression of fungal β-tubulin was significantly lower in
164 *Δpaba* as compared to all virus-free and virus-infected strains at 48 h (*P*= 0.02-0.002).

165 The expression levels of β-tubulin were not significantly different between the virus-free and virus-
166 infected couplets of CV and PV at both time points (*P*>0.05; Fig. 6A and 6B). However, fungal β-tubulin gene
167 expression “fungal load” in A78-infected as compared with the A78-free strain, was significantly higher at
168 48 h (*P*= 0.0075; Fig. 6C). This indicated that A78-infected isolate was capable of faster growth in *G.*
169 *mellonella* larvae than the A78-free isolate increasing the fungal burden.

170 **4. Discussion**

171 The main results of the study were as follows: (i) A dose of $1x10^5$ spores/larva was optimal for
172 determination of differences in survival rates between virus-free and virus-infected *A. fumigatus* isogenic
173 lines. (ii) There were no significant differences between the survival rates of CV-free/-infected and PV-free/-
174 infected *A. fumigatus* isogenic lines. (iii) The survival rates of moth larvae infected with A78-free isolate was
175 significantly higher than those infected with A78-infected isolate. (iv) The A78-infected *A. fumigatus* isolate
176 had a faster growth rate on AMM as compared to the A78-free isolate. (v) The fungal load determined by
177 qPCR was significantly higher in the A78-infected *A. fumigatus* isolate as compared to the A78-free isolate.

178 Numerous reports on optimizing the concentration of *A. fumigatus* spores for use in *G. mellonella* larvae
179 pathogenicity testing have been documented (Jackson et al., 2009; Slater et al., 2011). However, the current
180 study was the first to test mycovirus infected *A. fumigatus* isolates and it was necessary to determine the
181 optimal dosage for virulence of different isolates. As compared with previous studies on *A. fumigatus* done
182 by Slater et al., (2013) and Fallon et al.,(2011), a lower dose of spores were shown to be superior for
183 determining differences between virus-free and virus-infected couplets. This finding would imply that
184 mycovirus infections are able to alter fungal growth, hence a lower initial concentration of spores can still
185 become lethal post-infection.

186 Many studies which have used the invertebrate infection model have provided additional aspects of
187 fungal pathogenesis. The efficiency and compatibility of the moth infection model has been verified in
188 several studies on important human fungal pathogens (Brennan et al., 2002; Mylonakis et al., 2005; Reeves
189 et al., 2004). The advantages and limitations of this infection model have been reviewed extensively in
190 relation to vertebrate and other invertebrate models (Desalermos et al., 2012; Arvanitis et al., 2013). The
191 virulence of *A. fumigatus* mutants appear to be identical in both murine and *G. mellonella* infection models
192 (Slater et al., 2011; Vaknin et al., 2014). Thus, our investigations on the effects of mycoviruses on virulence
193 in the fungus have confirmed the utility and reliability of the moth model. The latter, is an inexpensive and
194 easy to manipulate alternative to the murine model, giving reproducible results and promises to be an

195 invaluable tool for further investigations on fungal virulence. Assessment of the fungal load has been used
196 commonly in antifungal drug studies using both mouse (Bowman et al., 2001) and *G. mellonella* infection
197 models (Mesa-Arango et al., 2012). Recently it has been shown that *A. fumigatus* conidia can grow in the
198 hemolymph of *G. mellonella* (Gomez-Lopez et al., 2014). From the obtained data presented here, it might
199 be stated that the *G. mellonella* has the ability of showing no virulence, different or equal virulence and also
200 reduced virulence as in PBS injected condition, in virus-free and virus-infected *A. fumigatus* injected
201 conditions and in the *Δpaba* isolate injected condition, respectively. In conclusion *G. mellonella* is a useful
202 model that can produce results compatible with other model organisms employed for investigation of *A.*
203 *fumigatus* pathogenesis in the presence of mycovirus infection.

204 Mycoviruses are increasingly being reported in a wide range of major fungal groups, including animal
205 and plant pathogens (van Diepeningen et al., 2008). While many of them have no or few obvious effects on
206 their host fungi, some do elicit phenotypic alterations including hypovirulence and host debilitation or less
207 frequently hypervirulence (Ghabrial and Suzuki, 2009). Hypovirulence effects have been shown to occur in
208 many fungal species including several important plant pathogens e.g. *Sclerotinia sclerotiorum* (Boland,
209 1992), *Botrytis cinerea* (Castro et al., 2003; Potgieter et al., 2013), *Cryphonectria parasitica* (Choi and Nuss,
210 1992; Nuss, 2005) and *Fusarium virguliforme* (Marvelli et al., 2014). Hypovirulence is of great interest due
211 to its potential for biocontrol of fungal diseases (Nuss, 2005). An association between the presence of
212 dsRNA and hypovirulence has been documented for several *Aspergillus* species (Elias and Cotty, 1996; van
213 Diepeningen et al., 2008). In Aspergillus, it also has been shown that phenotypic alterations such as growth
214 rate and pigmentation can be the result of RNA silencing of mycoviruses (Hammond et al., 2008). In *A.*
215 *fumigatus*, where CV and PV infections caused significant phenotypic effects, reduced growth rate and
216 hypovirulence, no effect on murine pathogenicity or fungal burden were caused by chrysovirus infection
217 (Bhatti et al., 2011b). Although the effects of PV on the phenotype of the fungus such as pigmentation and
218 sectoring were shown previously, effects on pathogenicity were not tested (Bhatti et al., 2011b). In the
219 current study the effect of PV infection was investigated for the first time and revealed that PV infection did
220 not alter the pathogenicity of *A. fumigatus* in *G. mellonella*. In the present investigation, our findings on CV

221 infection are in agreement with previous results obtained using the murine model. This suggests that *G.*
222 *mellonella*, whose immune system shares fundamental properties with mammalian immune system, is a
223 useful infection model to assess the effects of mycoviruses on *A. fumigatus*. The previous observations on
224 CV infection have largely been confirmed and extended in this investigation.

225 There are examples of beneficial effects of dsRNA mycoviruses on their hosts such as *Phytophthora*
226 *infestans* dsRNA virus-2 (PiRV-2), a mycovirus of the phytopathogen *P. infestans*, which causes devastating
227 diseases of potato and tomato. Here it was found that PiRV-2 infection is associated with enhanced
228 sporulation and high aggressiveness as compared to a virus-free isogenic line (Cai and Hillman, 2013).
229 Similarly increased activity in sporulation, pigmentation and high levels of virulence has also been shown in
230 the presence of 6.0 kbp dsRNA mycovirus in *N. radicicola* (Ahn and Lee, 2001). Infection of *A. fumigatus*
231 with A78 mycovirus caused a significant increase in radial growth and virulence in the moth model.
232 Although, AMM contains the minimum ingredients for fungal growth, the A78 virus-infected isolate grew
233 faster than the virus-free one in this medium. The increase in radial growth in both types of media, even
234 though it can be observed more clearly in AMM than ACM, would suggest that A78 virus infection confers
235 significant advantages for the growth of the host. The significant increase in growth and virulence in the
236 moth model are suggestive of hypervirulence. The hypervirulent nature of A78 virus was also supported at
237 the molecular level following assessment of the fungal burden as assessed by qPCR. In the future, the
238 hypervirulent nature of the A78 isolate should be tested using the murine model.

239 The potential of mycoviruses in regulating the pathogenicity of their hosts may provide a significant
240 treatment alternative at the early stages of human fungal infection.

241

242 **Acknowledgements**

243 We thank Dr Elaine Bignell for the non-virulent *A. fumigatus Δpaba* isolate and her scientific advices,
244 John Readman for his help on curing virus infection, Professor Mustafa Djamgoz and Dr Mine Güzel for

245 critical reading and comments. Selin Özkan thanks the Turkish Government Education program for
246 supporting her PhD investigations.

247

248 **References**

249 Ahn, I. P., Lee, Y. H., 2001. A viral double-stranded RNA up regulates the fungal virulence of *Nectria*
250 *radicicola*. Mol. Plant Microbe Interact. 14, 496-507.

251 Altincicek, B., Vilcinskas, A., 2006. Metamorphosis and collagen-IV-fragments stimulate innate immune
252 response in the greater wax moth, *Galleria mellonella*. Dev. Comp. Immunol. 30, 1108-1118.

253 Andrejko, M. et al. 2009. Antibacterial activity *in vivo* and *in vitro* in the hemolymph of *Galleria mellonella*
254 infected with *Pseudomonas aeruginosa*. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 152, 118-123.

255 Arvanitis, M. et al. 2013. Invertebrate models of fungal infection. Biochimica et Biophysica Acta 1832,
256 1378-1383.

257 Aufauvre-Brown, A. et al. 1998. Comparison of virulence between clinical and environmental isolates
258 of *Aspergillus fumigatus*. Eur. J. Clin. Microbiol. Infect. Dis. 17, 778-780.

259 Balajee, S. A. et al. 2005. *Aspergillus lentulus* sp. nov., a new sibling species of *A. fumigatus*. Eukaryot
260 Cell 4, 625-632.

261 Barratt, R. W. et al. 1965. Wild-type and mutant stocks of *Aspergillus nidulans*. Genetics 52, 233-246.

262 Bhatti, M. F. et al. 2011a. Complete nucleotide sequences of two dsRNAs associated with a new
263 partitivirus infecting *Aspergillus fumigatus*. Arch. Virol. 156, 1677-1680.

264 Bhatti, M. F. et al. 2011b. The effects of dsRNA mycoviruses on growth and murine virulence of
265 *Aspergillus fumigatus*. Fungal Genet. Biol. 48, 1071-1075.

266 Bhatti, M. F. et al. 2012. Incidence of dsRNA mycoviruses in a collection of *Aspergillus fumigatus* isolates.
267 Mycopathologia 174, 323-326.

268 Boland, G.J. 1992. Hypovirulence and double-stranded RNA in *Sclerotinia sclerotiorum*. Can. J. Plant
269 Pathol. 14, 10-17.

270 Bowman, J.C. et al. 2001. Quantitative PCR assay to measure *Aspergillus fumigatus* burden in a murine
271 model of disseminated aspergillosis: demonstration of efficacy of caspofungin acetate. Antimicrob.
272 Agents Chemother. 45, 3474-3481.

273 Brennan, M. et al. 2002. Correlation between virulence of *Candida albicans* mutants in mice and *Galleria*
274 *mellonella* larvae. FEMS Immunol. Med. Microbiol. 34, 153-157.
275 Brown, J. S. et al. 2000. Signature-tagged and directed mutagenesis identify PABA synthetase as essential
276 for *Aspergillus fumigatus* pathogenicity. Mol. Microbiol. 36, 1371-1380.
277 Cai, G., Hillman, B.I., 2013. Phytophthora viruses. Adv. Virus Res. 86, 327- 350.
278 Castro, M. et al. 2003. A double-stranded RNA mycovirus confers hypovirulence-associated traits to
279 *Botrytis cinerea*. FEMS Microbiol. Lett. 228, 87-91.
280 Choi, G. H., Nuss, D. L., 1992. Hypovirulence of chestnut blight fungus conferred by an infectious viral
281 cDNA. Science 257, 800-803.
282 Dawe, A.L., Nuss, D., 2001. Hypoviruses and chestnut blight: exploiting viruses to understand and
283 modulate fungal pathogenesis. Annu. Rev. Genet. 35, 1-29.
284 Desalermos, A. et al. 2012. Selecting an invertebrate model host for the study of fungal pathogenesis.
285 PLoS Pathol. 8, e1002451. doi:10.1371/journal.ppat.1002451.
286 Diaz-Ruiz, J. R., Kaper, J. M., 1978. Isolation of viral double-stranded RNAs using a LiCl fractionation
287 procedure. Prep. Biochem. 8, 1-17.
288 Elias, K. S., Cotty, P. J. 1996. Incidence and stability of infection by double-stranded RNA genetic elements
289 in *Aspergillus* section *flavi* and effects on aflatoxigenicity. Can. J. Bot. 74, 716-725.
290 Fallon, J. P. et al. 2011. Pre-exposure of *Galleria mellonella* larvae to different doses of *Aspergillus*
291 *fumigatus* conidia causes differential activation of cellular and humeral immune responses. Virulence
292 2, 413-421.
293 Fallon, J. et al. 2012. *Galleria mellonella* as a model for fungal pathogenicity testing. Meth. Mol. Biol.
294 845, 469-485.
295 Fuchs, B. B. et al. 2010. Methods for using *Galleria mellonella* as a model host to study fungal
296 pathogenesis. Virulence 1, 475-482.
297 Ghabrial, S. A., Suzuki N., 2009. Viruses of plant pathogenic fungi. Ann. Rev. Phytopathol. 47, 353-384.

298 Gomez-Lopez, A. et al. 2014. An invertebrate model to evaluate virulence in *Aspergillus fumigatus*: The
299 role of azole resistance. Med Mycol 52, 311-319.
300 Hammond, T.M. et al. 2008. Aspergillus mycoviruses are targets and suppressors of RNA silencing.
301 Eukaryot Cell 7, 350-357.
302 Harding, C. R. et al. 2012. *Legionella pneumophila* pathogenesis in the *Galleria mellonella* infection
303 model. Infect. Immun. 80, 2780-2790.
304 Hyder, R. et al. 2013. Two viruses of *Heterobasidion* confer beneficial, cryptic or detrimental effects to
305 their hosts in different situations. Fungal Ecol. 6, 387-396.
306 Jackson, J.C. et al. 2009. Conidiation color mutants of *Aspergillus fumigatus* are highly pathogenic to the
307 heterologous insect host *Galleria mellonella*. PLoS One 4, e4224.
308 Jamal, A. et al. 2010. Complete nucleotide sequences of four dsRNAs associated with a new
309 chrysovirus infecting *Aspergillus fumigatus*. Virus Res. 153, 64-70.
310 Jian, J. et al. 1997. Association of distinct double-stranded RNAs with enhanced or diminished virulence in
311 *Rhizoctonia solani* infected potato. Mol. Plant-Microbe Interact. 10, 1002-1009.
312 Latge, J. P., 1999. *Aspergillus fumigatus* and aspergillosis. Clin. Microbiol. Rev. 12, 310-350.
313 Marquez, L. M. et al. 2007. A virus in a fungus in a plant: three-way symbiosis required for thermal
314 tolerance. Science 315, 513-515.
315 Marvelli, R.A. et al. 2014. Identification of novel double-stranded RNA mycoviruses of *Fusarium*
316 *virguliforme* and evidence of their effects on virulence. Arch Virol 159, 349-352.
317 Mesa-Arango, A.C. et al. 2012. The non-mammalian host *Galleria mellonella* can be used to study the
318 virulence of the fungal pathogen *Candida tropicalis* and the efficacy of antifungal drugs during infection
319 by this pathogenic yeast. Med. Mycol. 51, 461-472.
320 Mowlds, P., Kavanagh K. 2008. Effect of pre-incubation temperature on susceptibility of *Galleria*
321 *mellonella* larvae to infection by *Candida albicans*. Mycopathologia 165, 5-12.
322 Mylonakis, E. et al. 2005. *Galleria mellonella* as a model system to study *Cryptococcus neoformans*
323 pathogenesis. Infect. Immun. 73, 3842-3850.

324 Nuss, D. L., 2005. Hypovirulence: mycoviruses at the fungal-plant interface. Nat. Rev. Microbiol.
325 3, 632-642.
326 Pontecorvo, G. et al. 1953. The genetics of *Aspergillus nidulans*. Adv. Genet. 5, 141-238.
327 Potgieter, C.A. et al. 2013. A wild-type *Botrytis cinerea* strain co-infected by double-stranded RNA
328 mycoviruses presents hypovirulence-associated traits. Virol J 10, 220.
329 Refos, J. M. et al. 2013. Double-stranded RNA mycovirus Infection of *Aspergillus fumigatus* is not
330 dependent on the genetic make-up of the host. PLoS One 8, e77381.
331 Reeves, E.P. et al. 2004. Correlation between gliotoxin production and virulence of *Aspergillus fumigatus*
332 in *Galleria mellonella*. Mycopathologia 158, 73-79.
333 Schmitt, M.J., Breinig, F., 2006. Yeast viral killer toxins: lethality and self-protection. Nature Rev. Microbiol.
334 4, 212-221.
335 Slater, J. L. et al. 2011. Pathogenicity of *Aspergillus fumigatus* mutants assessed in *Galleria mellonella*
336 matches that in mice. Med. Myc. 49, S107–S113.
337 Vaknin, Y. et al. 2014. The three *Aspergillus fumigatus* CFEM-domain GPI-anchored proteins (CfmA-C)
338 affect cell-wall stability but do not play a role in fungal virulence. Fung. Gen. Biol. 63, 55-64.
339 van Diepeningen, A. D. et al., 2008. Mycoviruses in the Aspergilli. In: Varga, J., Samson, R. A., (eds),
340 Aspergillus in the genomic era, Wageningen Academic Publishers, Wageningen, The Netherlands, pp.
341 133-176.
342 van de Sande, W. W. et al. 2010. Mycoviruses: future therapeutic agents of invasive fungal infections
343 in humans? Eur. J. Clin. Microbiol. Infect. Dis. 29, 755-763.
344 Varga, J. et al. 1998. Double-stranded RNA mycoviruses in species of *Aspergillus* sections *Circumdati*
345 and *Fumigati*. Can. J. Microbiol. 44, 569-574.
346

347 **TABLE AND FIGURE LEGENDS**

348

349 **Table 1**
350 *Aspergillus fumigatus* strains investigated in this study.
351
352
353
354 **Fig. 1.** The dsRNA profiles of *Aspergillus fumigatus* mycoviruses. Extracted dsRNAs were electrophoresed
355 on 1% agarose gel stained with ethidium bromide. The genome of *A. fumigatus* chrysovirus consists of
356 four dsRNA elements (1). *A. fumigatus* partitivirus-1 is a bipartite virus whose genome includes two dsRNA
357 segments (2). The dsRNA profile of A78 (3). White arrows indicate the dsRNA elements of each mycovirus.
358 Lane M shows the DNA ladder.

359

360 **Fig. 2.** Colony morphologies of virus-free and virus-infected *Aspergillus fumigatus* isolates. Mycovirus
361 infection may alter pigmentation, sectoring and growth of the fungus. All isolates grown on ACM 5 days
362 after inoculation as viewed from the front.

363

364 **Fig. 3.** Determination of the optimal spore concentration of *Aspergillus fumigatus* for pathogenicity
365 testing in *Galleria mellonella*. *G. mellonella* larvae were injected with conidiospores of *A. fumigatus* strains
366 A78-infected (A) and PV-free (B) using $1x10^7$, $1x10^6$, $1x10^5$ and $1x10^4$ conidiospores *per* larva. Infected
367 larvae were then incubated at 37 °C and monitored daily for 5 days. At the dose of $1x10^7$ spores per larva
368 the fungus killed almost all larvae dramatically within 24 h in both strains. It was impossible to see any
369 difference between the two strains at a dose of $1x10^6$ spores per larva, whereas a concentration of $1x10^5$
370 spores per larva was optimal to observe any possible differences in pathogenicity of *A. fumigatus* strains
371 as indicated above. Control experiments were conducted as untouched larvae (UTC), pierced larvae (PC)
372 and PBS-injected larvae (PBS).

373

374 **Fig. 4**. Mean survival of *Galleria mellonella* larvae infected with PBS (control) or $1x10^5$ spores /larva of
375 virus-free and virus-infected *Aspergillus fumigatus* isolates over a 5 day incubation period. The *A.*

376 *fumigatus Δpaba* strain was used as a non-virulent control in each experiment while the nominations of

377 the rest of the strains investigated are shown in Table 1. Survival curves were plotted and statistically

378 analyzed according to Kaplan-Meier estimation using the GraphPad Prism 6.0 program and *P* values were

379 estimated using Log rank and Wilcoxon tests. Error bars represent standard error values.

380

381

382 **Fig. 5.** Growth rate of isogenic lines of A78-free and A78-infected *A. fumigatus* isolates were noted and

383 measured on both on ACM and AMM plates. Equal numbers of spores were inoculated onto ACM and

384 AMM plates and the fungal phenotype examined 5 days after inoculation. Radial growth was measured

385 daily in three replicate plates. The means of the colony diameters measured were plotted to observe any

386 differences between the isolates on AMM (A) and ACM plates (B).

387

388 **Fig. 6.** Quantitative PCR showing fungal burden in virus-free and virus-infected *A. fumigatus* isolates at 6h

389 and 48h post infection. Cycle of threshold (Ct) values indicate the cycle where the DNA amplification was

390 first detectable. While expression of actin gene was measured in the PBS injected larvae, no β-tubulin

391 gene expression was observed. Expression levels of moth actin and fungal β-tubulin gene are shown in

392 the CV-free/infected couplet (A), in the PV-free/infected couplet (B) and in the A78-free/infected couplet

393 (C).

394

395 **Fig. S1.** Conventional PCR used to confirm *A. fumigatus* isolates. Fungal DNA were isolated using DNeasy

396 Blood and Tissue Kit according to the manufacturer's protocol (Qiagen, Valencia, CA) and PCR was

397 performed using universal ITS1 and ITS4 primers. Amplicons were electrophoresed on 3% agarose gel

398 stained with SYBRSafe and the expected amplicon size (597 bp) for *A. fumigatus* was detected (Lanes 1, 3

399 and 11: DNA marker; Lane 2: No template control; Lane 4: *A. fumigatus* isolate A78-free; Lane 5: *A.*

400 *fumigatus* isolate A78-infected; Lane 6: *A. fumigatus* PV-free; Lane 7: *A. fumigatus* PV-infected; Lane 8: *A.*

401 *fumigatus* CV-free; Lane 9: *A. fumigatus* CV-infected, Lane 10: *A. fumigatus Δpaba* isolate).